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Synthesis in *Escherichia coli* of Avian Reovirus Core Protein σA and Its dsRNA-Binding Activity

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The genome segment S2 of avian reovirus (ARV) S1133 was cloned and sequenced. The entire S2 nucleotide sequence is 1325 bp long with one long open reading frame that encodes a protein of 415 amino acids, corresponding to σA, a major core protein of ARV. S2 possesses a pentanucleotide, TCATC, at the 3′-terminus of its plus strand, common to other known genome segments of ARV and to 10 genome segments of mammalian reovirus. Amino acid sequence analysis revealed that σA contains a carboxy-terminal region (one-fourth of the protein) that is formed from α-helices and β-turns, and the remainder (three-fourths of the protein) is formed predominantly from β-strands and β-turns. Analysis of binding activity to poly(rI)–poly(rC)–agarose suggested that ARV protein A present in total virus-infected chicken embryo fibroblasts (CEF) had dsRNA-binding activity. To further characterize the binding activity, protein σA was subsequently expressed in *Escherichia coli* BL21(DE3) cells as a fusion protein and isolated by metal chelate affinity chromatography. The expressed protein eσA was further purified through a Superdex 75 HR 10/30 column after digestion of the purified fusion peptide with enterokinase. The expressed protein eσA has the same molecular weight as virion protein σA purified from ARV-infected CEF and is indistinguishable from virion protein σA by immunoblot analysis. The eσA binds cooperatively α ^{32}P-labeled dsRNA probe produced by run-off transcription of clone pGEM-3Zf(+)S4. The binding reaction is blocked by homologous ARV dsRNA or heterologous infectious bursal disease virus dsRNA and poly(rI)–poly(rC), but not by salmon sperm DNA. The results indicate that the expressed protein eσA has dsRNA-binding activity similar to that of σA obtained from infected cells, and its binding is sequence-independent.

INTRODUCTION

The structure and molecular composition of avian reovirus (ARV) are generally similar to those of mammalian reovirus (MRV), the prototype of the genus orthoreovirus (Gouvea and Schnitzer, 1982; Wickramasinghe *et al.,* 1993). Both groups have a genome consisting of 10 segments of double-stranded RNA (dsRNA) which is encapsidated by a double-shell capsid. However, ARV differs in its lack of hemagglutination activity and its ability to induce cell fusion (Robertson and Wilcox, 1986). Identification of all virus-encoded proteins and their coding assignments have been made (Varela and Benavente, 1994). Virus encoded at least 10 primary translation products which are separated into three size classes: large (λ), medium (μ), and small (σ). Eight of these products are structural proteins and the other two are nonstructural (Varela and Benavente, 1994). In contrast to MRV, ARV proteins have been much less characterized than their counterparts. By using monoclonal antibodies, protein σC has been indicated to be the target for type-specific neutralizing antibodies (Wickramasinghe *et al.,* 1993), while antibodies to σB are group-specific (Takehara *et al.,* 1987; Shapouri *et al.,* 1996). Both proteins are also involved in the induction of cell fusion and may play an important role in viral pathogenesis (Ni and Ramig, 1993; Theophilos *et al.,* 1995). Another protein of ARV, σNS, encoded by the S4 gene (Chiu and Lee, 1997), has been identified as a single-stranded RNA (ssRNA)-binding protein (Yin and Lee, 1998), similar to its MRV counterpart σNS.

It has been shown that MRV σ2, encoded by the MRV S2 gene, binds to reovirus dsRNA and may play an important role in virus transcription and replication (Schiff *et al.,* 1988; Dermody *et al.,* 1991). No observations have been reported for ARV σA, which is encoded by the ARV S2 gene and is a counterpart of MRV σ2.

In a recent study, poly(rI)–poly(rC)–agarose was used to examine the presence of RNA-binding proteins in cytoplasmic extracts prepared from ARV-infected cells. Subsequently, the S2 gene segment of ARV strain S1133 was cloned and sequenced. The coding region for σA has been inserted into an *Escherichia coli* expression plasmid. This approach provided a sufficient quantity of σA in a soluble form to allow our *in vitro* additional studies on its biological properties.

RESULTS

Binding of ARV σA to poly(rI)–poly(rC)–agarose

To determine if the ARV proteins could bind dsRNA, cytoplasmic extracts prepared from ARV S1133- and

The nucleotide sequence data reported in this paper have been deposited with the GenBank database and have been assigned Accession No. AF104311.

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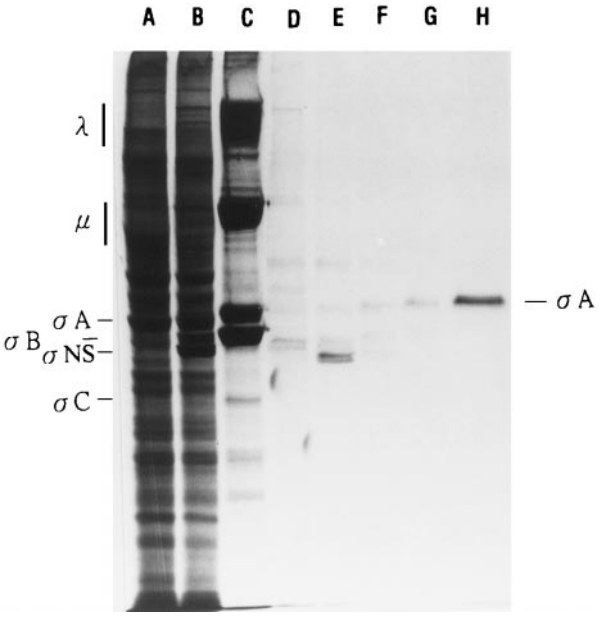

FIG. 1. Detection of poly(rI)–poly(rC)–agarose binding protein. Cytoplasmic extracts were prepared from ARV S1133- and mock-infected CEF cells labeled with [^{35}S]methionine. Fifty microliters of the sample was incubated with 100 μl of poly(rI)–poly(rC)–agarose. After centrifugation, proteins were then eluted from the pellets with binding buffer A containing various concentrations of NaCl and were analyzed on SDS–PAGE. Lanes D to H indicate the proteins eluted with binding buffer A-100, -200, -400, -600, and -1000 mM NaCl, respectively. The same volumes of the lysates from mock- and virus-infected ^{35}S-labeled CEF and ^{35}S-labeled virions were electrophoresed in lanes A, B, and C, respectively. The positions of bands corresponding to ARV peptides (Varela and Benavente, 1994) are indicated on the left. Samples eluted from poly(rI)–poly(rC)–agarose incubated with mock-infected CEF cells are not shown.

mock-infected CEF cells labeled with [^{35}S]methionine were incubated with poly(rI)–poly(rC)–agarose for 30 min at 23°C. After washing, proteins were eluted from agarose beads with binding buffer A containing various concentrations of NaCl and analyzed in SDS–PAGE. Figure 1 shows that a protein of approximately 45 kDa, which produced a single band eluted with binding buffer A–1000 mM NaCl, was observed in virus-infected cell lysates (Fig. 1, lane H), but not in mock-infected cell extracts (data not shown). The molecular size of this protein is consistent with that of the viral protein σA (Fig. 1, lanes B and C). Therefore, an ARV-coded protein with strong affinity for poly(rI)–poly(rC) could be protein σA, suggesting that ARV σA binds dsRNA. In addition to σA, no other ARV proteins bound to poly(rI)–poly(rC) were obtained.

Sequence analysis of the cloned S2 gene and the deduced sequence of σA

The complete nucleotide sequence of the S2 RNA is presented in Fig. 2 with the deduced amino acid sequence. The entire S2 gene sequence is 1325 nucleotides long. The distribution of the four bases was found to be relatively even: 22.34% A, 27.09% C, 24.76% G, and 25.81% T. The S2 gene contains one long ORF that starts with an ATG (at residues 16 to 18) and terminates at nucleotides 1264 to 1266 with a TAG codon. No additional ORF of significant length was observed in either the plus- or the minus-strand RNA. The start codon at position 16 to 18 has a strong context for initiation, with a purine at position −3 and a guanine at position +4 (Kozak, 1981); thus it is sufficient to encode a protein of 415 amino acid residues and a molecular mass of about 45.5 kDa, the size of viral protein σA as determined previously (Wickramasinghe *et al.,* 1993). The 3′-terminus contains a nucleotide sequence, TATTCATC, which is identical to those of the gene segments of known avian reovirus strains (Shapouri *et al.,* 1995; Yin *et al.,* 1997; Chiu and Lee, 1997; Le Gall-Recul *et al.,* 1999). The 5′-terminal sequence GCTTTTT differs from those of the S1 RNA (Shapouri *et al.,* 1996) and the S3 and S4 RNAs (Yin *et al.,* 1997; Chiu and Lee, 1997) of the same virus strains, whereas it is the same as that of the S3 RNA of a different virus strain from Muscovy duck (LeGall-Recul *et al.,* 1999). Such terminal motifs at both the 5′- and the 3′-termini were compared with those of MRV gene segments (Antczak *et al.,* 1982). The 3′-terminal pentanucleotide TCATC is found to be the sequence shared by the ARV and three serotypes of MRV. Also, there is no polyadenylation signal near the 3′ end of the S2 mRNA, like in the known segments of ARV and MRV.

A search made against the GenBank databases and ARV S1133 σA deduced amino acid sequence has shown that the degree of similarity is higher between ARV σA and MRV σ2 of three serotypes (Dermody *et al.,* 1991) than any other sequences and is 33, 32.8, and 34.3%, respectively. The σA contains a number of charged residues, of which the basic amino acids are predominant so that σA has a calculated isoelectric point of 8.62. The predicted amino acid sequence of σA showed that a region spanning residues 107–143 was notable as more consistently hydrophilic than the remainder of the protein (Kyte and Doolittle, 1982). The amino acids of this region also showed more consistently high probabilities of occurring on the protein surface than those in the remainder of σA (Emini *et al.,* 1985). Analysis of the possible secondary structure of σA indicated that the α-helices are not uniformly distributed and about 57% of them are in C-terminal one-fourth of the molecular (Nibert *et al.,* 1990). Amino acid residues 330 to 370 in this region are characterized by consistently high α-helix scores. In addition, this region contains the low scores of β-strand and β-turn. A small region at the extreme C-terminus of σA also has high α-helix scores. In contrast to the C-terminal one-fourth of σA, the remainder of the σA is characterized by alternations between regions of high β-strand and high β-turn scores. Several small regions of high α-helix scores were observed in this region. Al-

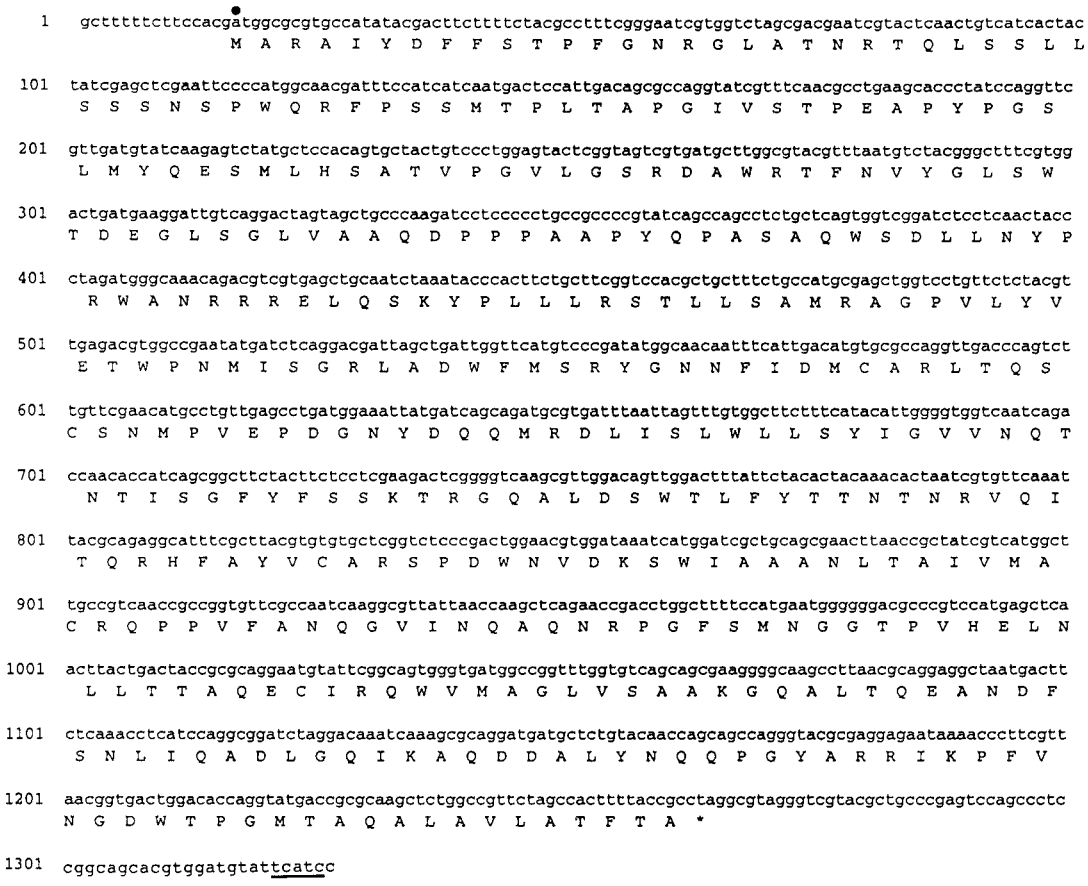

FIG. 2. The S2 segment RNA of ARV S1133 is presented in the DNA form along with the encoded σA amino acid sequence. The ORF starts at nucleotide 16 (●) and terminates at nucleotide 1266 (✳). The 3′-terminal pentanucleotide common to 10 segments of MRV and to S1, S2, and S4 RNAs of ARV is underlined.

though σA has three predicted glycosylation sites, it has been shown not to be a glycoprotein (Varela *et al.,* 1996). No potential phosphorylation site could be predicted for σA, in agreement with previous findings that none of the ARV proteins incorporates phosphorus during infection (Varela *et al.,* 1996).

Expression and purification of eσA

E. coli cells containing the pET 32a-σA construct were induced, and both cell lysate and insoluble protein fractions were examined on SDS–PAGE. The results revealed that a protein band of approximately 61 kDa was present in both fractions. Figure 3A (lane A) shows that this protein in a cell lysate was consistent with the molecular size of the expected fusion protein. The soluble fusion protein was then purified using a His-Bind Resin. Proteins in a fraction eluted with binding buffer B–100 mM imidazole produced a major single band with an estimated molecular size similar to the fusion protein (Fig. 3A, lane B). To obtain eσA for the functional assay, the purified fusion protein was digested with enterokinase and further purified using a Superdex 75 HR 10/30 column. The SDS–PAGE analysis of the purified protein demonstrated that the presence of a protein of approximately 45.5 kDa (Fig. 3A, lanes C and D) comigrated with σA (Fig. 3A, lane E). The identity of the expressed, purified protein eσA was further determined using a monospecific antiserum to the purified ARV σA. Western blot analysis indicated that bacterially expressed fusion protein showed a predominant band with the estimated molecular weight of 61 kDa (Fig. 3B, lanes A and B). Antiserum also reacted specifically against both the purified protein eσA and the purified virion σA, with an approximate molecular weight of 45.5 kDa (Fig. 3B, lanes C, D, and E). The results suggest that the purified eσA is encoded by the σA coding region of the ARV S2 gene.

Gel shift analysis

To determine if the purified protein eσA could bind nucleic acids, binding assays were carried out by incubating eσA with the labeled dsRNA probes. The incubation resulted in the formation of RNA–protein complexes that were altered as a function of protein concentration, implicating eσA in the gel shift activity (Fig. 4A). The binding of eσA to the labeled dsRNA probe was competed by unlabeled poly(rI)–poly(rC) (Fig. 4B). In both

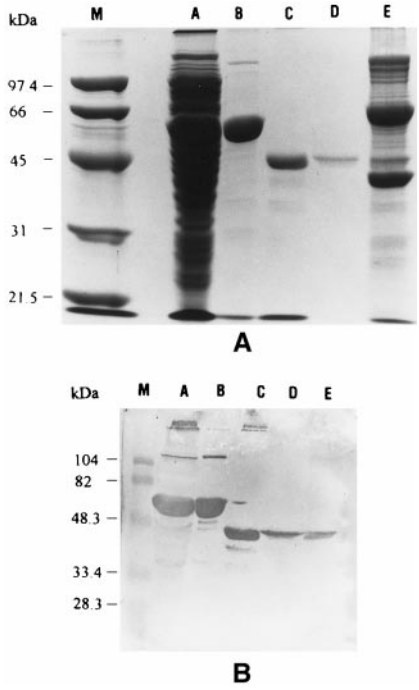

FIG. 3. Purification of protein eσA. (A) σA was expressed in *E. coli* BL21(DE3) as a fusion protein, purified, and stained with Coomassie blue after SDS–PAGE. Lane A is the sample in which the cells were induced with IPTG after a further 4 h, starting at 2 h after the OD_{600} of 0.6 was obtained, and lysed. Supernatant after centrifugation was used. Lane B, the sample obtained from the supernatant by purification with His-Bind Resin. Lane C, the sample obtained from the purified fusion protein as indicated in lane B after digestion with enterokinase. Lane D, the sample obtained from the protease-digested mixtures by further purification with Superdex 75 HR 10/30. Lane E, purified ARV S1133 virions. Molecular weight markers (Bio-Rad) are shown in lane M. (B) Immunoblot with a mouse anti-σA antiserum, 1:300, was performed for analysis of the reactivity specific to eσA. Lanes A to E are the same as for (A). Prestained molecular weight markers (Bio-Rad) are shown in lane M.

binding assays the RNA–protein complex formed was totally retarded and unable to migrate into the gel with no intermediate shifts noted, indicating a cooperative binding. Furthermore, no shift was noted with reactions in which the eσA had been heat treated or the reaction was amended with SDS or proteinase K (Fig. 4C). These results indicate that the shift of RNA mobility in the gel is from an interaction of RNA probe with eσA. Bovine serum albumin was also used to assess if nonspecific protein interactions could be the cause of shifting. Since no shift was apparent with this substitution, binding does not appear to be from nonspecific protein–nucleic acid interaction (Fig. 4C, lane F).

To ensure that the gel shift was not due to an aggregation of eσA, serial dilutions of eσA starting from lower concentration were used for testing. As eσA concentrations were increased in the standard binding reaction, a ladder-like profile of protein–RNA complexes as indicated in Fig. 5 was observed. The results suggest that the formation of discrete complexes is prior to the putative cooperative binding of the RNA probe.

Binding of eσA to dsRNA was not sequence-specific because RNA–protein complex formation was not observed regardless of whether the RNA competitor was homologous ARV or heterologous infectious bursal disease virus (IBDV) (Fig. 6) or poly(rI)–poly(rC) (Fig. 4B). Salmon sperm dsDNA was not able to compete (Fig. 6, lane D), indicating that eσA did not bind dsDNA. In addition, binding of eσA to dsRNA was not blocked when denatured ARV dsRNA was used as the competitor, suggesting that eσA may not bind ssRNA (Fig. 6, lane B).

Effect of ionic concentration on binding activity

The ability of eσA to interact with dsRNA under different ionic conditions was tested by increasing the NaCl concentrations in the binding buffer. Gel shift assay results showed that the binding of eσA to dsRNA was not significant at NaCl concentrations above 500 mM, and the optimal salt concentration for binding to eσA was 100 to 200 mM (Fig. 7).

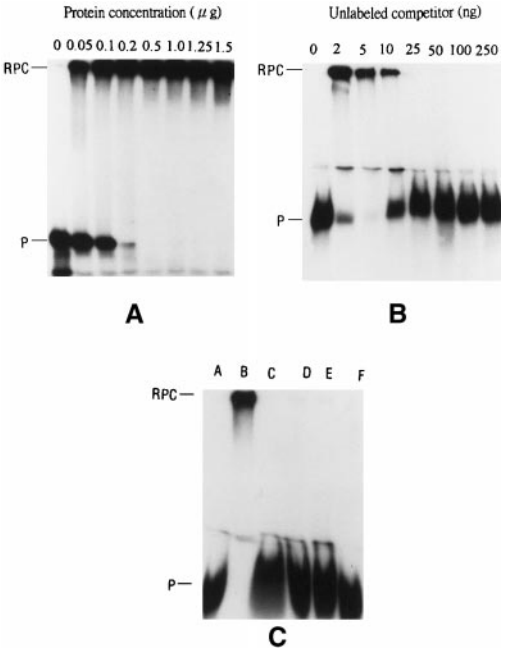

FIG. 4. Gel shift assay of eσA binding to dsRNA. Autoradiographs of binding reactions after electrophoresis through a native polyacrylamide gel are shown. (A) Binding reactions (as described under Materials and Methods) with ^{32}P-labeled dsRNA probe and eσA in the indicated concentrations. The positions of the unbound dsRNA probe (P) and the shifted RNA–protein complexes (RPC) are indicated on the left. (B) Binding reaction mixtures contained ^{32}P-labeled dsRNA probe, 25 ng eσA, and unlabeled poly(rI)–poly(rC) in the indicated concentrations. Competitor was incubated with eσA at 23°C for 30 min prior to addition of dsRNA probe. (C) Binding reactions with ^{32}P-labeled dsRNA probe and eσA (500 ng) were amended with 0.1% SDS (lane C) or proteinase K (1 μg) (lane D), or the eσA was heated to 100°C for 10 min prior to binding reactions (lane E). Lane A, ^{32}P-labeled dsRNA probe. Lane B, eσA (500 ng) with ^{32}P-labeled dsRNA probe. Lane F, BSA (500 ng) with ^{32}P-labeled dsRNA probe.

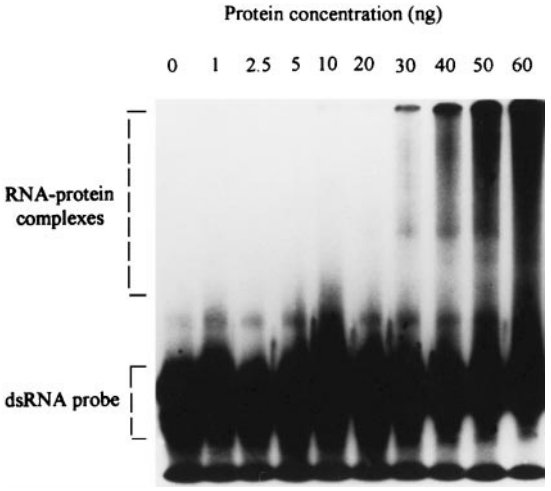

FIG. 5. Gel shift assay with eσA. Samples were analyzed by native gel electrophoresis and subjected to autoradiography for 36 h. Binding reaction (as described under Materials and Methods) containing ^{32}P-labeled dsRNA probe and eσA in concentrations as indicated.

DISCUSSION

To our knowledge, at least six proteins of MRV have RNA-binding affinity (Brentano *et al.,* 1998). Related activities of ARV proteins which are the counterparts of MRV are largely unknown. The results of this paper reveal that the bacterially expressed protein eσA, like authentic ARV σA, strongly binds dsRNA but in a non-sequence-specific manner.

Analysis of the S2 gene sequence indicates that there is one long ORF coding for a protein the same size as the known S2 gene product, the major core protein σA (Schnitzer, 1985; Wickramasinghe *et al.,* 1993), as evident

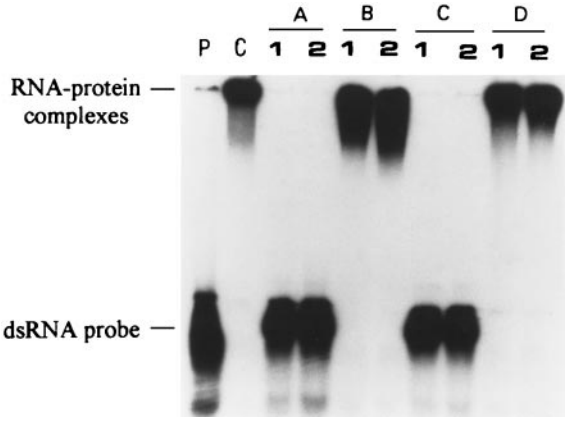

FIG. 6. Autoradiograph of nucleic acid binding specificity of eσA after electrophoresis through a native polyacrylamide gel. Binding reactions containing eσA (500 ng) and ^{32}P-labeled dsRNA probe were amended with unlabeled competitor RNA 250 ng (lane 1) or 2000 ng (lane 2) prior to incubation. Homologous ARV dsRNA (A) and denatured ARV dsRNA (B) and heterologous IBDV dsRNA (C) and salmon sperm DNA (D) were used as unlabeled competitors. P, dsRNA probe. C, dsRNA probe with eσA.

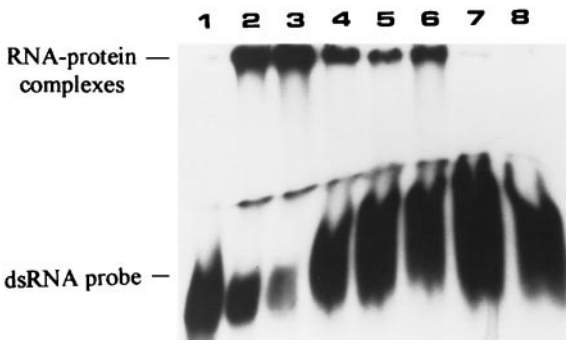

FIG. 7. Autoradiograph of the effect of salt concentrations on nucleic acid binding by eσA. Binding reactions were carried out over a range of NaCl concentrations. Lanes 2 to 8, eσA (500 ng) and ^{32}P-labeled dsRNA probe binding reactions with NaCl concentrations ranging from 100 (lane 2) to 700 mM (lane 8), in 100 mM increments. Lane 1, ^{32}P-labeled dsRNA probe.

by determining the identity of the expressed protein eσA using immunoblot assay. The results suggest a monocistronic nature for the S2 gene of ARV, with the translation of σA from this ORF which corresponds to translation of 94% of the S2 gene. This would confirm previous findings (Varela and Benavente, 1994) that σA, like other ARV proteins except the small size σC, does correlate with full-length translation of the genomic segments. Comparison of the terminal sequences among the four known small size gene segments shows that they do share the conserved 10-nucleotide sequences at the 3′-terminus within their plus strands. The pentanucleotide sequence TCATC within this conserved sequence is common to 10 MRV plus strands. The 3′-terminal sequence of the plus strand is expected to play a significant role during virus replication (Antczak *et al.,* 1982). Thus, the presence of this conserved sequence at their 3′-termini suggests that the viruses may share a similar mechanism for RNA synthesis.

Examination of the deduced amino acid sequence of σA indicated that σA appeared to contain a carboxy-terminal region (one-fourth of the protein) that contains α-helices and β-turns and a larger amino-terminal region (three-fourths of the protein) that is predominantly β-strands and β-turns, as predicted by using the techniques of Nibert *et al.* (1990). These results are very similar to those for MRV σ2 described previously (Dermody *et al.,* 1991), indicating that both proteins ARV σA and MRV σ2 may have similar functions. Although sequence analysis of the ARV σA does not reveal any conserved RNA-binding motifs or regions that are similar and may play a role in RNA binding, in fact our results from the analysis of binding activity to poly(rI)–poly(rC) revealed that ARV-infected cells contained a protein that comigrated with ARV σA and had the dsRNA-binding activity (Fig. 1). In this experiment, other than σA, no signals corresponding to σB and λC, the counterparts of MRV σ3 and λ1, respectively, were observed. Since both

MRV σ3 and λ1 have been shown to be the dsRNA-binding proteins (Huismans and Joklik, 1976; Schiff *et al.,* 1988), it would need to be determined if ARV σB and λC bind dsRNA or if the optimal conditions for their binding differ. In addition, when the α-helical prediction at residues 354 to 374 of MRV σ2 was positioned on a helical wheel (Schiffer and Edmundson, 1967), it appeared to form an α-helix that is distinctly amphipathic and may be involved in protein–protein interaction involving another reovirus protein(s) (Dermody *et al.,* 1991). However, ARV σA does not contain such amphipathic structure.

The dsRNA-binding properties of ARV σA were further characterized using the bacterially expressed protein eσA. The entire coding region of σA was readily amplified and its encoded protein was expressed with the pET 32a expression system in a fusion manner to increase amounts of the protein in soluble form. To ensure that the 16-kDa peptide which was fused to σA did not affect the RNA–protein binding, eσA was further purified to homogeneity through a Superdex column after the treatment of the fusion protein with enterokinase (Fig. 3). By using the purified eσA in native gel shift assays, the RNA–eσA complex remained in a free or a bound state (Fig. 4A). The assay of the resolution of individual RNA–protein complexes showed the sequential formation of a ladder of RNA–protein complexes at low protein concentration (Fig. 5). In addition, our results also indicated that dsRNA–eσA protein complexes formed over a large range of ionic concentrations (Fig. 7). Together, the results suggested that the formation of RNA–protein complex is a cooperative binding, which can be explained by a high affinity of the eσA for dsRNA. The feature characteristic of cooperative binding for eσA to dsRNA described here is very similar to those described for the ssRNA binding of tomato spotted wilt virus N protein (Richmond *et al.,* 1998) and the dsRNA binding of hantavirus N protein (Gott *et al.,* 1993) and vaccinia virus E3 protein (Ho and Schuman, 1996), all of which have been characterized in cooperative binding assays. Previous work has indicated that MRV σ2 weakly bound dsRNA as indicated by Northwestern blotting (Schiff *et al.,* 1988; Dermody *et al.,* 1991). The effect of ionic concentration on binding activity was tested using the same technique (Dermody *et al.,* 1991). The optimal conditions for the dsRNA binding of MRV σ2 were between 0 and 50 mM NaCl. In comparison, these results are different from those obtained in our study, in which ARV eσA strongly bound to dsRNA has been characterized and a large range of ionic concentrations is optimal for binding reaction (Fig. 7). This could be due to the different techniques used for the binding assays. The specificity of eσA binding to dsRNA was confirmed by competition assay. Our results indicated that the binding of eσA to dsRNA probe was competed by poly(rI)–poly(rC) (Fig. 4B), dsRNA of ARV, and IBDV, but not by salmon sperm dsDNA (Fig. 6). Thus, eσA bound specifically to dsRNA in a sequence-independent manner, which has been characterized as a common feature for several virus dsRNA-binding proteins (Huisman and Joklik, 1976; Boyle and Holmes, 1986; Ho and Shuman, 1996).

Although the functions of MRV σ2 are largely unknown, a temperature-sensitive (*ts*) mutant of MRV T3D whose mutation maps to the S2 gene (*ts* 447) synthesizes approximately 5% of the wild-type level of ssRNA and assembles virions which contain only 0.1% of the normal amount of dsRNA when grown at the restrictive temperature (Ramig *et al.,* 1978). Furthermore, Northwestern blotting assays have shown that MRV σ2 binds MRV dsRNA (Schiff *et al.,* 1988; Dermody *et al.,* 1991). These observations suggest that MRV σ2 may play an important role in viral RNA synthesis or in packaging the viral genome segments during virion assembly. ARV σA, like MRV σ2, has been shown to be a viral core protein (Schnitzer *et al.,* 1982; Wickramasinghe *et al.,* 1993). The recent data revealed that both the expressed protein eσA and the protein σA obtained from ARV-infected cells had dsRNA-binding activity, similar to that of MRV σ2 (Schiff *et al.,* 1988; Dermody *et al.,* 1991). This binding activity may play a similar role in the ARV transcription and replication as described for MRV. This study represents an initial step in the characterization of the protein σA. The deduced amino acid sequences of σA reported here will allow us to insight more into its structure and function.

MATERIALS AND METHODS

Cell and virus

ARV strain S1133, a commercially available vaccine strain (Vineland Laboratories), has been adapted for growth in chicken embryo fibroblasts (CEF) and plaque-purified twice previously (Wu *et al.,* 1994).

Labeling of ARV-infected cells and ARV particles with [^{35}S]methionine

The procedures for labeling of ARV-infected cells and ARV particles with [^{35}S]methionine were essentially as described previously (Yin and Lee, 1998). Briefly, CEF monolayers were infected with ARV S1133 or mock-infected with medium alone (M199 supplemented with 2% fetal calf serum) and labeled with [^{35}S]methionine (Amersham Life Science; sp act >1000 Ci/mmol; 100 μCi in 5 ml Dulbecco's modified Eagle's medium without methionine). The cells were lysed, and cytoplasmic extracts were prepared and dialyzed against binding buffer A–100 mM NaCl (2 mM HEPES, pH 7.4, 5 mM magnesium acetate, 0.1% NP-40, 1 mM dithioerythritol, 100 mM NaCl, 10% glycerol). To obtain radiolabeled virions, equal amounts of [^{35}S]methionine were added to virus-infected CEF as described above. All infections were allowed to continue for 25 h. Cells were then harvested and radio-

labeled virions were purified as described (Yin and Lee, 1998).

Binding of cytoplasmic proteins to poly(rI)–poly(rC)–agarose

To identify ARV proteins with associated RNA-binding activity, 50 μl of cytoplasmic extracts prepared above were incubated with 100 μl of poly(rI)–poly(rC)–agarose (Pharmacia) which had been washed three times with binding buffer A–100 mM NaCl at 23°C for 30 min with gentle agitation. The agarose was pelleted. Proteins were then eluted with 100 μl of binding buffer A-100, -200, -400, -600, and -1000 mM NaCl. The nature of the proteins in each eluate was analyzed by SDS–PAGE (Laemmli, 1970) after precipitation with 10% 1,1,2-trichloroacetic acid.

Cloning and nucleotide sequencing of ARV S2 gene

The techniques for extraction of dsRNA from virions and isolation of individual S2 gene segments were those described previously (Lee *et al.,* 1994). The procedures for the preparation of double-stranded cDNA (dscDNA) and the cloning of cDNA into the *Pst*I site of the plasmid pBR 322 were essentially the same as described elsewhere (Yin *et al.,* 1997; Chiu and Lee, 1997). Briefly, S2 RNA was polyadenylated at the 3′ end and the dscDNA synthesized using a commercial cDNA synthesis kit (BRL). The product was ligated into the *Pst*I site of pBR322 and subsequently used to transform *E. coli* JM 103. Transformants that were resistant to tetracycline and sensitive to ampicillin were randomly selected. Specific clones for S2 RNA were determined by Northern blot hybridization, subcloned into pUC 18, and sequenced by the methods of Sanger *et al.* (1977), using DNA Sequenase (U.S. Biochemicals).

pET 32a-σA construction and fusion protein expression

To express ARV σA for its functional assay, the open reading frame coding for σA in S2 gene segment was amplified and placed into *E. coli.* The forward primer (5′-GTATAGGATCCATGGCGCGTGCCATATAC-3′) corresponded to the 5′ region of the σA gene and incorporated a *Bam*HI restriction site immediately upstream of the initiation codon of the ORF on the S2 gene segment. The reverse primer (5′-GACTATCTCGACCTAGGCGGTAAAAGTGG-3′) was complementary to the 3′ end of the σA gene and contained a *Xho*I restriction site immediately downstream of the termination codon. Both primers were used to amplify the σA coding region with *Taq* polymerase. The construction of pET 32a-σA by insertion of the amplified product into the pET 32a bacterial expression vector (Novagen) was made essentially as described previously (Yin *et al.,* 1997). pET 32a-σA expressed a 61.5-kDa fusion protein, which included all 415 amino acids encoded by the S2 gene and a 16-kDa protein encoded by the vector itself, at the N-terminal end of the fusion peptide. The procedures for fusion protein expression were essentially those described previously (Yin *et al.,* 1997). To analyze the whole bacterial cell samples, pellets from culture cells (DE3) induced with 1 mM IPTG for 4 h were resuspended in 1× Laemmli loading buffer and boiled for 3 min prior to gel analysis (Laemmli, 1970).

Purification of the expressed protein eσA

To obtain soluble or insoluble expressed fusion proteins, whole cell pellets from the bacterial cultures were disrupted by sonication at 4°C in a binding buffer B (Tris–HCl, pH 7.9, 100 mM NaCl), and the homogenates were centrifuged at 360,000 *g* for 30 min. The pelleted insoluble materials were resuspended in 1× Laemmli loading buffer and boiled for 3 min before gel analysis. The supernatant was collected and the soluble fusion proteins were purified using a His-Bind Resin column (Novagen) according to the manufacturer's instruction manual. After washing with the same binding buffer B, the proteins were eluted with 5 ml of binding buffer B–60 mM, –100 mM, and –1000 mM imidazole, respectively. The nature of the samples eluted was then analyzed by SDS–PAGE. The fusion protein-containing fraction was dialyzed against enterokinase cleavage buffer (20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 2 mM $CaCl_2$) overnight with several changes and enterokinase (Novagen) was added at a final concentration of 3 U. The reaction mixture was incubated for at least 16 h at 21°C. The expressed protein eσA was subsequently purified by passing through a Superdex 75 HR 10/30 Column (Pharmacia), based on the manufacturer's instruction manual.

DNA construct and *in vitro* transcription

To prepare the ^{32}P-labeled virus-specific dsRNA probe by run-off transcription, a recombinant plasmid pGEM-3Zf(+) S4 was constructed. The forward primer (5′-GGGCGAATTCGAGGGGTCGCCACA-3) was identical to nucleotides 1141 to 1154 of the S4 mRNA (numbered according to Chiu and Lee, 1997) and incorporated an *Eco*RI restriction site at the 5′ end; the reverse primer (5′-ACTCAAGCTTACTCAAGCTTGATGAATAAGAGTCC-3′) was complementary to the 3′ end of the S4 mRNA and contained a *Hin*dIII restriction site at its 5′ end. Both primers were used for *Taq* amplification using the ARV S4 cDNA clone (Chiu and Lee, 1997) as the template. The amplified products were digested with *Eco*RI and *Hin*dIII and then ligated into the corresponding sites of the pGEM-3Zf(+) *in vitro* transcription vector (Boehringer Mannheim) to construct a recombinant plasmid pGEM-3Zf(+)S4, which was used to transform DH5α. The expected nucleotide sequence of pGEM-3Zf(+)S4 was confirmed by dideoxynucleotide sequencing (Sanger *et*

al., 1977). Radiolabeled virus-specific dsRNA probes were generated by annealing complementary transcripts from the pGEM-3Zf(+)S4. The sense strand was obtained using SP6 RNA polymerase to produce a 62-nucleotide RNA from *Eco*RI-linearized pGEM-3Zf(+)S4. The complementary strand was transcribed by T7 RNA polymerase to produce a 62-nucleotide RNA from *Hin*dIII-linearized pGEM-3Zf(+)S4. Run-off products labeled with [α-^{32}P]CTP (3000 Ci/mmol; Amersham) were hybridized in 10 mM HEPES buffer, pH 7.5, containing 50 mM NaCl for 5 min at 95°C, followed by slow cooling to room temperature. To ensure that these hybrid RNAs were double stranded, they were treated with 0.5 μg/ml RNase A for 15 min at 37°C. After phenol/chloroform extraction and ethanol precipitation, the dsRNA probes were resuspended and used for binding reactions with eσA.

Gel shift analysis

Purified protein eσA and ^{32}P-radiolabeled dsRNA probes (10^4 to 10^5 cpm) were combined with a binding reaction buffer (150 mM NaCl, 1 mM DTT, 0.5% Tween 20, 20 mM HEPES, 5 mM MgOAc, 10% glycerol, pH 7.4) for a total reaction volume of 15 μl and incubated at 30°C for 15 min. The reaction mixtures were then separated by electrophoresis on 6% polyacrylamide nondenaturing gels (Konarska and Sharp, 1987) and subjected to autoradiography for 16–18 h unless indicated in some cases.

Blocking of eσA binding activity was analyzed by amending the above binding reactions with either 200 or 2000 ng of homologous RNAs (ARV dsRNA or denatured dsRNA), heterologous RNA (IBDV dsRNA) (Lee *et al.,* 1994), and salmon sperm DNA (Sigma), after incubation at 30°C for 15 min and continuing at 30°C for further 30 min. Denatured ARV dsRNA was prepared by adding formamide to ARV dsRNA at a final concentration of 50% of the volume, heating ARV dsRNA at 110°C for 10 min (DIGI-BLOCK; Laboratory Devices, Inc., U.S.A.) and then chilling on ice before use.

To determine ionic strength optima for RNA binding, binding reactions were modified to have NaCl concentrations ranging from 100 to 700 mM (in 0.1 M increments) and then incubated at 30°C for 15 min. Reaction mixtures were then separated by electrophoresis on 6% polyacrylamide nondenaturing gels and visualized by autoradiography.

Antiserum preparation and Western blotting

Monospecific antiserum against the purified ARV σA (Yin and Lee, 1998) was prepared in BALB/c mice. The animals were intraperitoneally injected with the purified σA emulsified in complete Freund adjuvant. Two subsequent boosts with the same proteins in incomplete Freund adjuvant were given every 2 weeks. Sera were collected at 2 weeks after final injection. Western immunoblotting was performed by using a 1:300 dilution of the above antiserum as described (Yin *et al.,* 1997).

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